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Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype

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Abstract

This study determined hydrogen emissions by beef cattle under different dietary conditions and how cattle genotype and rumen microbial community affected emissions. Thirty-six Aberdeen Angus- (AAx) and thirty six Limousin-sired (LIMx) steers received two diets with forage:concentrate ratios (DM basis) of either 8:92 (Concentrate) and 52:48 (Mixed). Eighteen animals of each genotype received each diet. Methane and H_2 emissions were measured individually in indirect respiration chambers. Hydrogen emissions (mmol/min) varied greatly throughout the day, being highest after feeding, and averaged about 0.10 mol H_2/mol CH_4. Hydrogen emissions were higher (mol/kg DM intake) with the Mixed diet. Methane emissions (mol/d and mol/kg DM intake) were higher from steers receiving the Mixed diet ($P < 0.001$); AAx steers produced more CH_4 on a daily (mol/d $P < 0.05$) but not on a DM intake (mol/kg DM intake) basis. Archaea ($P = 0.002$) and protozoa ($P < 0.001$) were more and total bacteria ($P < 0.001$) less abundant ($P < 0.001$) in the Mixed diet. Relative abundance of *Clostridium* Cluster IV was greater ($P < 0.001$) and Cluster XIVa ($P = 0.025$) less on the Mixed diet. Relative abundance of *Bacteroides* plus *Prevotella* was greater ($P = 0.018$) and *Clostridium* Cluster IV less ($P = 0.031$) in LIMx steers. There were no significant relationships between H_2 emissions and microbial copy number. It was concluded that the rate of H_2 production immediately after feeding may lead to transient overloading of methanogenic archaea capacity to use H_2, resulting in peaks in H_2 emissions from beef cattle.

Methane is a greenhouse gas with a global warming potential 25-fold that of CO_2[1]. Ruminant livestock production through the enteric fermentation of feed contributes significantly to greenhouse gas production by agriculture; in the United Kingdom, CH_4 accounted for 37% of all agricultural emissions in 2005[2]. Enteric production of CH_4 also represents a loss of energy (from 2 to 12% of gross energy (GE) intake)[3], which might otherwise be available for growth or milk production. Understanding the mechanisms of methanogenesis and the microorganisms involved is important for devising sustainable mitigation strategies to lower the environmental impact of ruminant livestock production.

Molecular H_2 plays an important role in intermediary metabolism in the rumen[4]. Hydrogen is formed by bacteria, protozoa and fungi from the fermentation of carbohydrate. Hydrogen and CO_2 are the principal substrates for methane formation by archaea[5,6]. Hydrogen is also a vital intermediate or substrate in other reactions. Ruminal interspecies H_2 transfer is a process that affects the metabolism of both the microbes that produce H_2 and those that utilise it[7]. Methanogenic archaea require some accumulation of H_2 to grow rapidly enough to prevent them washing out of the rumen[4]. On the other hand, the accumulation of H_2 exerts a thermodynamic inhibitory effect on H_2-producing organisms and causes the fermentation products of these and other microbial species to be changed[7]. As fibrolytic *Ruminococcus* spp. are H_2 producers (via acetate formation), their growth and consequently fibre degradation may be inhibited by H_2 accumulation[4,7]. These pure culture studies indicate that decreasing H_2 concentrations in the rumen would be doubly beneficial in terms of CH_4 emissions and fibre breakdown.

Several studies have measured H_2 concentrations in ruminal digesta, as reviewed by Janssen[4]. Hydrogen concentrations increase *in vitro* after adding feed, and the concentrations are diet-dependent. Fewer studies have reported H_2 emissions *in vivo*. In one study[8] involving two sheep, it was noted that the animals produced two-fold different amounts of CH_4: the sheep with lower CH_4 emissions produced more H_2. In another study using sheep, Takenaka et al.[9], concluded that H_2 emissions were on average 2.1% (vol:vol) of CH_4 emissions based on exhaled gas concentrations. There were periods of high H_2 emission when H_2 formation occurred at a faster rate than methanogenesis, particularly when concentrate feeds were included in the diet. Similar investigations in cattle have to the best of our knowledge not been published. The aim of the present study was therefore to measure both H_2

and CH_4 emissions from beef steers fed two contrasting finishing diets typical of production in the United Kingdom: a high concentrate diet based on barley and a mixed forage:concentrate diet including grass and whole crop barley silages, barley grain and maize distillers dark grains (similar to maize distillers grains with solubles).

Materials and Methods

This study was conducted at the Beef Research Centre of SRUC (6 miles south of Edinburgh, UK) in 2011. The experiment was approved by the Animal Experiment Committee of SRUC and was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986.

Animals, experimental design and diets

The seventy two cross-bred steers used were from a rotational cross between purebred Aberdeen Angus or Limousin sires and crossbred dams of those genotypes and referred to as AAx and LIMx, respectively. The steers were fed two complete diets using a forage wagon, consisting (g/kg DM) of either 480 forage: 520 concentrate (Mixed) or 75 forage: 925 concentrate (Concentrate). The composition of the diets and nutritional composition of the feeds are given in Tables 1 and 2 respectively.

Immediately before the experiment reported here, DM intake (DMI) and live-weight (LW) gain of the steers had been measured in a feeding trial for 8 weeks (to be reported elsewhere). The feeding trial was of a 2 × 2 factorial (genotype × diet) design with the steers being stratified by LW on entry. The experiment reported here was a continuation of the feeding trial and steers therefore continued on the diet they were fed during the feeding trial. Steers were allocated to the six respiration chambers over a 12-week period, using a randomised block design (6 chambers times 4 weeks) which was repeated three times. Within each block, each treatment of the 2 x 2 factorial (genotype × diet) experimental design was replicated once in each respiration chamber. Steers were allocated to blocks to minimise variation in LW (mean LW (kg) 674, SEM 4.2) on entry to the respiration chambers. Emissions from each of the 72 steers were therefore measured once as described below.

Respiration chamber design, operation and measurements

Six indirect open-circuit respiration chambers were used (No Pollution Industrial Systems Ltd., Edinburgh, UK). The total chamber volume (76 m^3) was ventilated by

recirculating fans set at 450 l/s. Air was removed from the chambers by exhaust fans set at 50 l/s giving approximately 2.5 air changes/h. Temperature and relative humidity were set at 15°C and 60% relative humidity respectively. Total air flow was measured by in-line hot wire anemometers which were validated by daily measurements made with an externally calibrated anemometer (Testo 417, Testo Ltd, Alton, Hampshire, UK). Temperature and humidity were measured using sensor probes in the exhaust air outlet (Johnson Controls, Milan, Italy) and atmospheric pressure, corrected for altitude, with a Vantage Pro2 weather station (Davis Instruments, Haywood, Ca, USA). Chambers were operated under negative pressure (50 N/m^2). Methane concentrations were measured by infrared absorption spectroscopy and H_2 by a chemical sensor (MGA3000, Analytical Development Co. Ltd., Hoddesdon, UK). The analyser was calibrated with a gas mixture of known composition. Gas concentrations were recorded for each chamber and for inlet air every 6 min. Prior to the beginning of the experiment, gas recoveries were measured by releasing CO_2 at a constant rate into each chamber. The mean recovery was 98% (SEM 3.0) which was not different from 100%.

To accustom the steers to the chamber environment, 6 d prior to chamber measurements groups of steers were moved to the building in which chambers were located and loose-housed in single pens (4 × 3 m) of identical design to pens within the chambers. After 6 days, the steers were then moved to the chambers and remained there for 72 h, with CH_4 and H_2 measurements recorded in the final 48 h used in the analysis. Steers were fed once daily and weight of feed within the bins was recorded at 10 s intervals using load cells. Front doors of chambers were briefly opened at about 08.00 h daily to remove feed bins and again to replace bins with fresh feed at approximately 09.00 h. The pens were cleaned daily between 08.00 and 09.00 h. Exact times when doors were opened were recorded.

Rumen sampling and volatile fatty acid (VFA) analysis

Immediately after the steers (within 2 h) left the respiration chambers, samples of rumen fluid were obtained (one per animal) by inserting a tube (16 × 2700 mm Equivet Stomach Tube, Jørgen Kruuse A/S, Langeskov, Denmark) nasally and aspirating manually. Approximately 50 ml fluid were strained through two layers of muslin and stored at -20 °C to await analysis. Samples for VFA analysis (1 ml) were deproteinised by adding 0.2 ml metaphosphoric acid (215 g/litre) and 0.1 ml internal

125 standard (10 ml 2-ethyl n-butyric acid /litre) and VFA concentrations determined by
126 HPLC[10]. For DNA analysis, 5 ml strained rumen fluid were mixed with 10 ml
127 phosphate buffered saline containing glycerol (30% v/v) and stored at -20 °C.
128
129 *DNA analysis*
130 DNA extraction was carried out using a method based on repeated bead beating plus
131 column filtration[11]. DNA concentrations were determined with a NanoDrop ND
132 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA
133 was diluted to 0·5 ng/µl in 5 µg/ml herring sperm DNA for amplification with
134 universal bacterial primers UniF and UniR and 5 ng/µl in 5 µg/ml herring sperm DNA
135 for amplification of other groups[12]. qPCR was carried out using a BioRad iQ5 as
136 described by Ramirez-Farias et al.[13]. Calibration curves were prepared on three
137 separate batches in different qPCR runs. Bacterial primer sets, methods development
138 and target species may be found in Ramirez-Farias et al.[13]. Template DNA from
139 *Roseburia hominis* A2-183 (DSM 16839^T) was used for bacterial calibration.
140 Archaeal amplification was using the primers described by Hook et al.[14] and
141 calibrated using DNA extracted from *Methanobrevibacter smithii* PS, a gift from M.P.
142 Bryant, University of Illinois. Protozoal 18S rRNA gene amplification was calibrated
143 using DNA amplified from bovine rumen digesta with primers 54f and 1747r[15].
144 Coverage of qPCR primers was checked from original references and by use of the
145 Probe Match tool of the Ribosome Database Project[16].
146
147 *Feed analysis*
148 Feed samples were analysed for DM, ash, crude protein, acid detergent fibre, neutral
149 detergent fibre, starch[17] and GE by adiabatic bomb calorimetry.
150
151 *Calculations and statistical analysis*
152 To minimize bias caused by entry of air when doors were opened for feeding and, as
153 during this period (54 min. SD 22.5) steers did not have access to feed, gas
154 concentrations measured during this period were not used for further analysis. Instead,
155 and to minimize bias, these values were replaced by the mean value of measurements
156 (n=10) made in the last hour before doors were opened. If a steer had consumed food
157 during that period, mean values for the hour preceding feed consumption were used.
158 All data, including gas concentrations, air flow, temperature, humidity, atmospheric

pressure and records for feed consumption, were loaded into a database. Dry air flow was calculated and corrected to standard temperature and pressure for each individual record of gas concentration. Daily gas production was then calculated as the average of individual values.

Measurements were not made on one steer because of illness and data were rejected from three steers because of an air leak in one chamber; these consisted of two LIMx steers fed the Concentrate diet, one LIMx steer fed the Mixed diet and one AAx steer fed the Mixed diet. Data were analysed using Genstat (Version 11.1 for Windows, VSN Int. Ltd., Oxford, UK) using linear mixed models where the fixed factors were the 2 × 2 arrangement of genotype and diet, and random factors, block and chamber. Since samples for VFA analysis were available for only seven weeks of the experiment, these data were analysed as a 2 × 2 factorial arrangement of genotype and diet with week of experiment and chamber. Data are reported as means and SED unless otherwise stated. Multiple linear regression models were fitted to predict CH_4 and H_2 emissions from the whole dataset. Fitted terms included *Clostridium* Cluster IV, XIVa, *Bacteroides* + *Prevotella*, archaea and protozoa (expressed as copy number/ng DNA). To help with variable selection, all subsets of predictors were examined, with subsets compared using adjusted R-squared and Akaike's Information Criterion (AIC).

Results

Cattle offered the Mixed diet consumed less feed (Table 3) whether expressed as total daily DM intake (DMI, $P < 0.001$) or as g/kg LW ($P = 0.009$) than cattle offered the Concentrate diet. DMI was also greater ($P = 0.002$) for AAx than for LIMx steers.

Whether expressed as mol/d, mol/kg DMI or kJ/MJ GE intake (GEI, Table 3), steers fed the Concentrate diet produced less CH_4 than steers fed the Mixed diet ($P<0.001$). AAx steers produced more CH_4 (mol/d $P=0.032$) than LIMx steers but this difference disappeared when CH_4 production was expressed relative to DMI or GEI.

Hydrogen production from the steers was on average 0.10 mol H_2/mol CH_4 (Table 3). There was a significant diet × genotype interaction such that Concentrate-fed AAx steers produced less total H_2 than LIMx steers but the opposite was found for the Mixed diet. When expressed as mol/kg DMI or kJ/MJ GEI, there was no

193 interaction, and Mixed diet-fed steers produced more H_2 than Concentrate-fed steers.
194 However as a proportion of CH_4 production (mol H_2/mol CH_4), Concentrate-fed
195 steers produced more H_2 than Mixed-fed steers ($P < 0.001$).
196 Fig. 1 shows an example, comprising one steer fed the Concentrate and one
197 fed the Mixed diet, of changes in the rate of CH_4 and H_2 production (mmol/min) over
198 a 24-h period after fresh feed was offered. There were intermittent peaks, particularly
199 in H_2 emission rates throughout the day. Aligning these peaks with records of feed
200 consumption, it was apparent that the peaks in CH_4 and H_2 concentrations occurred a
201 short time after feed consumption. Further analysis showed that whereas median H_2
202 production rates (0.63 vs 0.68 mmol/min, SED 0.060, Concentrate v Mixed), did not
203 differ ($P > 0.05$) between diets, the frequency of H_2 production more than 0.5
204 mmol/min above median values (0.053 v 0.117, SED 0.210, $P < 0.001$) was greater
205 for Mixed- than Concentrate-fed steers. Thus, a substantial part of the greater H_2
206 output in Mixed-fed steers (mol/kg DMI) was related to peaks in H_2 concentration
207 associated with feeding.
208 Molar proportions (mmol/mol, Table 4) of acetic ($P < 0.001$), butyric ($P =$
209 0.013) and valeric acids ($P = 0.01$) were greater and those of propionic acid ($P <$
210 0.001) less in rumen fluid samples from Mixed than Concentrate-fed animals.
211 Genotype had no effect on VFA proportions.
212 Both diet and genotype influenced microbial numbers (Table 5). The
213 Concentrate diet supported lower copy numbers of archaea ($P = 0.002$) and protozoa
214 ($P < 0.001$) but larger copy numbers of total bacteria ($P < 0.001$) than the Mixed diet.
215 *Clostridium* Clusters IV and XIVa and *Bacteroides* + *Prevotella* accounted for
216 between 0.7 and 0.8 of copy numbers represented by total bacteria and there were no
217 differences in this proportion due to diet or genotype. The relative abundance of
218 *Clostridium* Cluster IV (proportion of total bacteria, Table 5) was greater ($P < 0.001$)
219 and that of *Clostridium* Cluster XIVa ($P =0.025$) was less on the Mixed diet than the
220 Concentrate diet ($P < 0.001$). Proportionally, AAX steers supported larger copy
221 numbers of *Clostridium* Cluster IVa (P=0.031) and lower numbers of *Bacteroides* +
222 *Prevotella* ($P = 0.018$).
223 There was a significant correlation between H_2 and CH_4 production (mol/kg
224 DMI) for the Mixed but not the Concentrate diet (Fig. 2). For the Mixed diet, linear
225 regression analysis found a significant slope (0.088, SE 0.0041, $P < 0.001$) with
226 intercept not different from 0. No microbial predictors were able to explain a

significant amount of variability in H_2 emissions between individual animals. For CH_4 (mol/kg DMI), there was a relationship ($r^2 = 0.30$) with copy numbers (x 10^3/ng DNA) of archaea and Clostridium cluster XIVa: CH_4 (mol/g DMI) = 1.07 - 0.00298 Cluster XIVa (s.e. 0.00083, $P = 0.001$) + 0.0094 Archaea (s.e. 0.0024, $P < 0.001$)

Discussion

Enteric fermentation in animals occurs predominantly in the absence of oxygen. Under such conditions, microbial communities adapt differently to the disposal of the reducing equivalents that are generated by glycolysis. Some microorganisms use an internal redox mechanism, such as in the formation of propionate and succinate. However, most microbial fermentation results in the formation of molecular H_2. The fate of H_2 depends on the animal species and its anatomical configuration. In man, with a relatively rapid gut transit time, reductive acetogenesis ($H_2 + CO_2 \rightarrow$ acetate) and H_2 gas tend to predominate as mechanisms for disposal of H_2. About 50% of human subjects in Europe also produce CH_4; CH_4 production competes with other metabolic processes but H_2 gas is still produced in these subjects[18]. Hydrogen emissions from ruminants are known to be proportionally much smaller and CH_4 emissions much greater[19]. Van Zijderfeld et al.[20] measured H_2 production from dairy cows hourly for 9 h and reported greater concentrations when nitrate was included in the diet but, to the authors' knowledge, this is the first report in which total daily H_2 emissions by cattle have been quantified on a large scale using indirect respiration chambers.

Hydrogen emissions

Previous studies have reported lower H_2 concentrations for ruminants fed all-forage diets than for diets containing various proportions of concentrate and forage whether measured as concentrations of H_2 dissolved in rumen fluid[21], in the rumen gas phase[22] or in exhaled air[9]. There do not appear to be any reports of H_2 emissions for high-concentrate diets in live animals. Here, daily H_2 emissions were similar with both diets and genotypes, but when converted to units per DM intake, H_2 production was greater on the Mixed than on the Concentrate diet. Total daily H_2 emissions were about 1% and 10% of CH_4 emissions on a mass and molar basis respectively. A total H balance was constructed from estimates of the amounts of carbohydrate fermented in the rumen and observed mean VFA molar proportions for each diet. Whilst the

amount of H_2 produced per unit carbohydrate fermented on the Concentrate diet was less than on the Mixed diet (3.6 v 4.9 moles H_2/mole carbohydrate fermented), estimates of total H_2 produced were not dissimilar between diets (169 v 177 moles/day, Concentrate v Mixed) because of both the lower fermentability (due to the presence of fermentation end-products in the silages) and the lower daily feed intakes of the Mixed diet. Thus H_2 emissions accounted for less than 2% of estimated total H_2 production from fermentation. Further, after accounting for H consumed in synthesis of microbial biomass, total recovery of hydrogen in microbial biomass, H_2 and CH_4 was similar between diets (108 and 114% of H produced for Concentrate and Mixed diets) indicating that there were no major H-consuming processes unaccounted for or that differed between diets.

Peaks in H_2 emission rates (Fig. 1) were observed after feed was consumed and these peak H_2 emission rates were greater on the Mixed diet. Increases in H_2 emission rates after feeding are consistent with measurements in sheep of H_2 concentrations in rumen fluid[21,23], rumen head-space gas[22,24] and respiration chambers[25,26]. The larger size of the meal-related peaks in H_2 emissions on the Mixed diet accounted for the differences in daily H_2 emissions (g/kg DMI) observed for this diet. One might have expected that there would be correlations between the ruminal microbiota and H_2 emissions particularly the balance between ciliate protozoa and *Clostridium* Cluster IV as major H_2 producers and archaea as consumers, but no relationships between H_2 emissions and any of the different groups of micro-organisms were found. It is possible that the primers used may not have detected all H_2 producing bacteria. Alternatively, the differences between diets in H_2 emissions are more likely to be related to the nature of the diets fed and the consumption patterns of individual cows. First the peaks in H_2 emissions may be caused by physical displacement of gas from the rumen head space by the feed consumed[27]. Because the Mixed diet contained larger proportions of long forage and had a higher moisture content (443 v 853 g DM/kg fresh weight), the bulkier Mixed diet may have caused greater displacement of rumen head space gas and hence greater H_2 emissions. Secondly, compared to the Concentrate diet, the Mixed diet contained higher concentrations of more slowly fermented cell wall carbohydrates and less starch and also higher concentrations of soluble feed constituents derived from the silages fed, particularly amino acids and fermentation products. Therefore there may be increased production of H_2 from rapid fermentation of soluble feed components

immediately after consumption of the Mixed diet which exceeded the capacity of methanogens to utilise the H_2. The peaks in H_2 emissions after consuming feed were also more defined and discrete than the peaks in CH_4 emissions (Fig. 1). A possible explanation for this is that while CH_4 is an end-product of metabolism of H_2 by archaea, the H_2 present in the ruminal gas phase can either be emitted by eructation or can redissolve in ruminal fluid and be utilised for CH_4 production by the archaea[28]. This may also explain the poor relationship between CH_4 and H_2 emissions (Fig. 2), as H_2 emissions will depend not only on rates of production by H_2-generating metabolism exceeding the capacity of archaea to consume H_2 but also the rate at which dissolved/gaseous H_2 is utilised. Both of these will depend on the meal size and rate of feed consumption of individual animals.

Methane production

As found in other studies[2,29], CH_4 production (mol/d) was substantially lower when the diet containing more than 900 g concentrate/kg DM was fed compared to the mixed forage:concentrate diet, thus confirming the well-established strategy of reducing CH_4 emissions by increasing the concentrate proportion of the diet. Mean methane yields (MJ/MJ GEI) were 0.039 and 0.062 for the Concentrate and Mixed diets respectively. These compare with values of 0.030 ("for diet containing more than 900 kg concentrates / kg DM") and 0.065 ("for all other diets") adopted by IPCC[1] for estimating CH_4 emissions. Thus values predicted from IPCC[1] for CH_4 production for the Mixed diet differed little from those observed (predicted v observed; 298 v 287 litre/d). However IPCC[1] predictions underestimated CH_4 production from the Concentrate diet (predicted v observed, 155 v 200 litre/d). The reason for the higher CH_4 production for the Concentrate diet in the current experiment was probably that the cereal fed was barley rather than maize. When high-concentrate diets based on maize and barley were fed to feedlot cattle[28], CH_4 production of 0.028 and 0.040 of GEI were reported for maize and barley respectively. Similarly, CH_4 production of 0.033 and 0.046 of GEI were reported for maize and barley-based concentrates (800 g concentrate /kg DM) albeit in different years[30]. Finally, CH_4 values of 0.04 of GEI for a barley-based diet (900 g/kg diet DM[31], and recently 0.03 of per GEI for a maize-based concentrate[32] have been reported. Thus, the value suggested by IPCC[1] of 0.030 for high concentrate diets is probably inappropriate for diets based on barley and 0.04 per GEI might be more

appropriate. The reasons for the difference between barley and maize have been discussed[29,32] and are most likely due to the more rapid and complete fermentation of barley grain in the rumen and the higher fibre concentration in barley. The simple approach used by IPCC[1] does not account for variations in diet digestibility or differences in the efficiency of utilisation of absorbed nutrients for productive purposes. Methane emissions from the present study were estimated relative first to ME (estimated from feed analysis) intake as a proxy for digestibility and secondly with respect to steer LW gain during the feeding trial which preceded this experiment. For the Concentrate diet, estimates were 0.058 MJ CH_4 /MJ ME intake and 6.5 moles CH_4/kg LW gain compared to 0.101 and 11.7 for the Mixed diet. Relative to the Concentrate diet, the Mixed diet produced 1.74- (ME basis) and 1.80-fold (LW gain basis) more CH_4 in comparison with 1.58- fold expressed on a GE basis. Thus the difference in CH_4 emissions between diets is amplified when expressed on a ME or LW gain basis.

Although total daily CH_4 emissions were greater for AAx steers, this difference was accounted for by differences in DM intake. Thus CH_4 emissions (mol/kg DMI) did not differ between the similar genotypes, although there were effects of individual sires[33].

Diet and microbial numbers

Analysis of the rumen microbial community provided information about how diet affected the main groups of bacteria, total ciliate protozoa and archaea. The three groups of bacteria were chosen to represent the main groups of bacteria (*Firmicutes* and *Bacteroidetes*) that are known to colonise the rumen[34-36], but it should be noted that the primers used would not account for all species of *Firmicutes* or *Bacteroidetes.* The three groups of bacteria accounted for more than 0.70 of total bacteria copy numbers and this proportion was not influenced by diet or genotype. The *Clostridium* groups form part of the *Firmicutes* phylum, which are usually more abundant than *Bacteroidetes* in rumen samples[34-36] and this was true for the AAx but not LIMx steers in this experiment. Part of the variation in relative abundance (proportion of total bacteria) of the two *Clostridium* Clusters was due to diet. Cluster IV, encompassing the highly cellulolytic *Ruminococcus* and several *Eubacterium* spp.[37] were more abundant with the Mixed diet. The Cluster XIVa grouping, whose abundance was lower in the Mixed diet, would contain *Butyrivibrio* and related

spp[37], none of which are known to possess the ability to break down crystalline cellulose[38]. Ciliate protozoa were more numerous with the Mixed diet, a result which seems to be at odds with the general observation that adding concentrate to a forage diet usually increases protozoal numbers[19,39]. There is a limited number of reports on the rumen microbial community when diets containing high proportions of concentrate were fed. The abundance of archaea increased when concentrate was increased from 100 to 500 g/kg diet[40] and decreased when dietary concentrate was increased from 500 to 900 g/kg[41] (similar to the present experiment). However when Popova et al.[42] compared starch and fibre-rich concentrates in a diet containing 870 g concentrate /kg there were no difference in numbers of methanogens between diets. When dietary concentrates were increased[43] from 0 to 700 g/kg, increasing concentrate reduced the numbers of *Fibrobacter succinogenes* and increased the numbers of genus *Prevotella* but there were no differences between diets in the populations of *Ruminococcus albus* or *R. flavefaciens.* This is in contrast to the decrease in C*lostridium C*luster IV and no change in *Bacteroides* plus *Prevotella* numbers when concentrate was increased in the present study. Similarly, increases in protozoal numbers were reported[42,43] when concentrate or dietary starch was increased, again in contrast to the decrease in numbers reported here and elsewhere[31]. These differences are probably explained by the different dietary protocols and approaches to community analysis used in the experiments. For example Carberry et al[43] compared 0 and 700 g concentrate /kg whilst the comparison was between 500 and 920 g concentrate /kg in the present study.

In terms of our focus on H_2 emissions, it was perhaps surprising that the H_2-producing *Ruminococcus* spp. of Cluster IV and total protozoa which produce abundant H_2[44] were not more correlated with CH_4, as H_2 is the main substrate for methanogenesis in the rumen[28,45]. There is no obvious explanation, except perhaps that any effect of the abundance of H_2 producers was swamped by effects of long-term adaptation to the diets fed. Alternatively, a more detailed taxonomic description within the groups, best derived from metagenomic information, might identify key genera and species that dictate H_2 production and thereby influence methanogenesis.

Many researchers believe, and some studies are beginning to show, that the host animal exerts a controlling effect on its own gut microbiota[46-48]. The findings here that the relative abundance of *Bacteroides* plus *Prevotella* was less and cluster IV greater in AAx than LIMx steers on the corresponding diets would support such a

hypothesis and may provide a mechanism for the greater feed intakes observed with the AAx steers.

Implications

Recently, when interactions between H_2 and other gases in the atmosphere were considered[49], it was proposed that H_2 is an indirect greenhouse gas with a global warming potential of 5.8 compared to 25 for CH_4 on a carbon dioxide mass equivalent basis. On a daily basis, total (CH_4 plus H_2) mean emissions from enteric fermentation were 3.6 and 5.1 kg CO_2 for the Concentrate and Mixed diets of which H_2 contributed 12 and 13 g CO_2 daily. Thus, although inefficiency of capture of H_2 during inter-species H_2 transfer is a loss of energy from the system, in terms of overall greenhouse gas production by ruminants, its contribution will be negligible with the exception of circumstances where methanogenesis is severely disrupted, e.g. when halogenated compounds are used to inhibit methanogenesis[25].

In conclusion, this large-scale study of the effect of diet, feeding pattern and cattle genotype on H_2 emissions by cattle has revealed that H_2 emissions can be up to 10% on a molar basis of CH_4 emissions from beef cattle on commonly used diets. Most H_2 was produced shortly after feeding, and the concentration followed that of CH_4. However the feeding-related increases in H_2 were not related to the microbial populations and therefore are more likely due to between-diet differences in feeding patterns and the nutrients rapidly fermented upon feed ingestion. Cattle genotype affected H_2 emissions *via* differences in feed intake and this may be related to differences in microbial community structure. The observations are consistent with the review by Janssen[4] that the capacity for archaeal methanogenesis is in balance with rates of H_2 production, such that some accumulation of H_2 is required for methanogenesis to occur. The quantities of H_2 emitted and the lower radiative forcing potential of H_2 suggest that H_2 emissions present a minor environmental problem in comparison with those of methane.

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Conflicts of interest

The authors declare no conflict of interest.

Authorship

T.W., R.J.W. and R.R. initiated the research. CAD, JJH, DWR. participated in planning and facilitating the animal work. N.McK. and S. M. de S. carried out DNA extraction and qPCR. J.A.R supervised the respiration chamber studies and wrote the manuscript with input from R.J.W. All authors provided feedback on the manuscript. The authors declare no conflict of interest.

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Table 1. Ingredient composition (fresh weight basis; g/kg) of high- concentrate and mixed forage: concentrate diets

Ingredient	High concentrate	Mixed forage:concentrate
Barley straw	81	0
Grass silage	0	413
Whole crop barley silage	0	340
Barley grain	688	156
Maize distillers dark grains	200	86
Molasses	20	0
Minerals-vitamin supplement*	10	5

*Contained (mg/kg): Fe, 6036; Mn, 2200; Zn, 2600; Iodine, 200; Co, 90; Cu, 2500; Se 30; (µg/kg): vitamin E, 2000; vitamin B12, 1000; vitamin A, 151515; vitamin D, 2500

Table 2. Chemical composition of feeds incorporated into high-concentrate and mixed forage: concentrate diets*

	Barley	MDDG	Silage	WCBS	Straw
DM(g/kg)	850	865	211	329	825
(g/kg DM)					
Ash	22	47	67	60	37
Crude protein	104	273	147	111	21
Acid detergent fibre	69	216	345	312	519
Neutral detergent fibre	163	377	567	540	826
Starch	592	22	6	141	3
pH			3.9	4.7	
Gross energy (MJ/kg DM)	18.8	21.8	19.0	19.1	17.1

Barley, barley grain; MDDG, maize distillers dark gains; silage, grass silage; WCBS, whole crop barley silage, Straw, barley straw.
*Molasses contained 688 g DM /kg and Gross Energy 15.3 MJ/kg DM

Table 3. Intakes, methane and hydrogen production from steers fed either a high concentrate or mixed forage:concentrate diets

(Means with SED for 17 observations per mean)

Diet	Concentrate		Mixed			Probability		
Genotype	AAx	LIMx	AAx	LIMx	SED	Genotype	Diet	GxD
DMI								
kg/d	11.4	10.0	10.2	8.7	0.52	0.002	<0.001	NS
g/kg LW	16.1	15.1	15.2	13.4	0.76	0.016	0.009	NS
Hydrogen								
mol/d	0.92	1.08	1.18	1.05	0.106	NS	NS	0.027
mol/kg DMI	0.084	0.112	0.116	0.122	0.0111	NS	0.006	NS
kJ/MJ GEI	1.27	1.66	1.74	1.84	0.168	NS	0.004	NS
Methane								
mol/d	9.4	8.5	13.6	12.0	0.72	0.032	<0.001	NS
mol/kg DMI	0.83	0.87	1.34	1.38	0.077	NS	<0.001	NS
kJ/MJ GEI	39.0	39.9	61.7	64.2	3.31	NS	<0.001	NS
H_2 :CH_4 mol/mol	0.101	0.126	0.086	0.088	0.0135	NS	<0.001	NS

Concentrate, high concentrate diet; Mixed, mixed forage: concentrate diet.
AA, Aberdeen Angus; LIM, Limousin; G x D, genotype x diet; DMI, dry matter intake; GEI, Gross Energy intake.

Table 4. Volatile fatty acid (VFA) molar proportions (mmol/mol) in rumen fluid samples obtained from steers fed either a high concentrate or mixed forage:concentrate diets

(Means with SED for 8 observations per mean)

Diet	Concentrate		Mixed			Probability		
Genotype	AAx	LIMx	AAx	LIMx	SED	Genotype	Diet	GxD
Acetic	557	562	670	670	27.9	NS	<0.001	NS
Propionic	290	306	172	173	34.9	NS	<0.001	NS
Butyric	105	92	114	125	13.4	NS	0.013	NS
Valeric	16	16	12	13	1.8	NS	0.010	NS
Branched chain	32	24	30	20	6.2	Ns	NS	NS

Concentrate, high concentrate diet; Mixed, mixed forage: concentrate diet.
AA, Aberdeen Angus; LIM, Limousin; G x D, genotype x diet; Branched chain: iso-butyric plus isovaleric acids

Table 5. Microbial numbers in samples of ruminal digesta

(Means with SED for 13 observations per mean)

Diet	Concentrate		Mixed			Probability		
Genotype	AAx	LIMx	AAx	LIMx	SED	Genotype	Diet	GxD
Archaea[1]	30.4	25.7	46.4	36.7	5.84	NS	0.002	NS
Protozoa[2]	37.2	40.0	102.1	71.4	16.1	NS	<0.001	NS
Total bacteria	669	761	492	513	57.7	NS	<0.001	NS
Clostridium								
Cluster IV[1]	138	122	179	135	32.7	NS	NS	NS
Cluster XIVa[1]	127	122	75	69	18.9	NS	<0.001	NS
Bacteroides plus *Prevotella*[1]	218	302	157	202	29.1	0.002	<0.001	NS
Relative abundance[3]								
Clostridium								
Cluster IV[1]	0.21	0.17	0.35	0.26	0.046	0.031	<0.001	NS
Cluster XIVa[1]	0.19	0.16	0.15	0.13	0.023	NS	0.025	NS
Bacteroides plus *Prevotella*[1]	0.33	0.40	0.32	0.40	0.041	0.018	NS	NS
Sum[3]	0.74	0.73	0.82	0.79	0.057	NS	NS	NS

Concentrate, high concentrate diet; Mixed, mixed forage: concentrate diet.
AAx, Aberdeen Angus cross; LIMx, Limousin cross; G x D, genotype× diet.
Results are expressed as copy numbers (x 10^3)/ng DNA as determined by qPCR of 16S rRNA[1] and 18S rRNA[2].
[3] Relative abundance as a proportion of total bacteria; sum is that of *Clostridium* Cluster IV plus Cluster XIVa plus *Bacteroides* plus *Prevotella*.

Legends for figures

Figure 1. Changes in methane (dashed line) and hydrogen (solid line) concentrations during a 24 h period (beginning after fresh feed offered at 09.00h). Examples are given for (a) one steer fed a high concentrate and (b) one steer fed a mixed forage:concentrate diet. Diets were fed *ad libitum* and solid bars denote when feed was consumed.

Figure 2 Relationships between daily hydrogen and methane (mol/kg DM intake) production for cattle fed either (a) a high concentrate (●) or (b) a mixed forage: concentrate (○) diet. Significant regression line is shown for the mixed forage:concentrate diet: ($y = 0.088x$; SE 0.0041; $P < 0.001$)

Figure 1

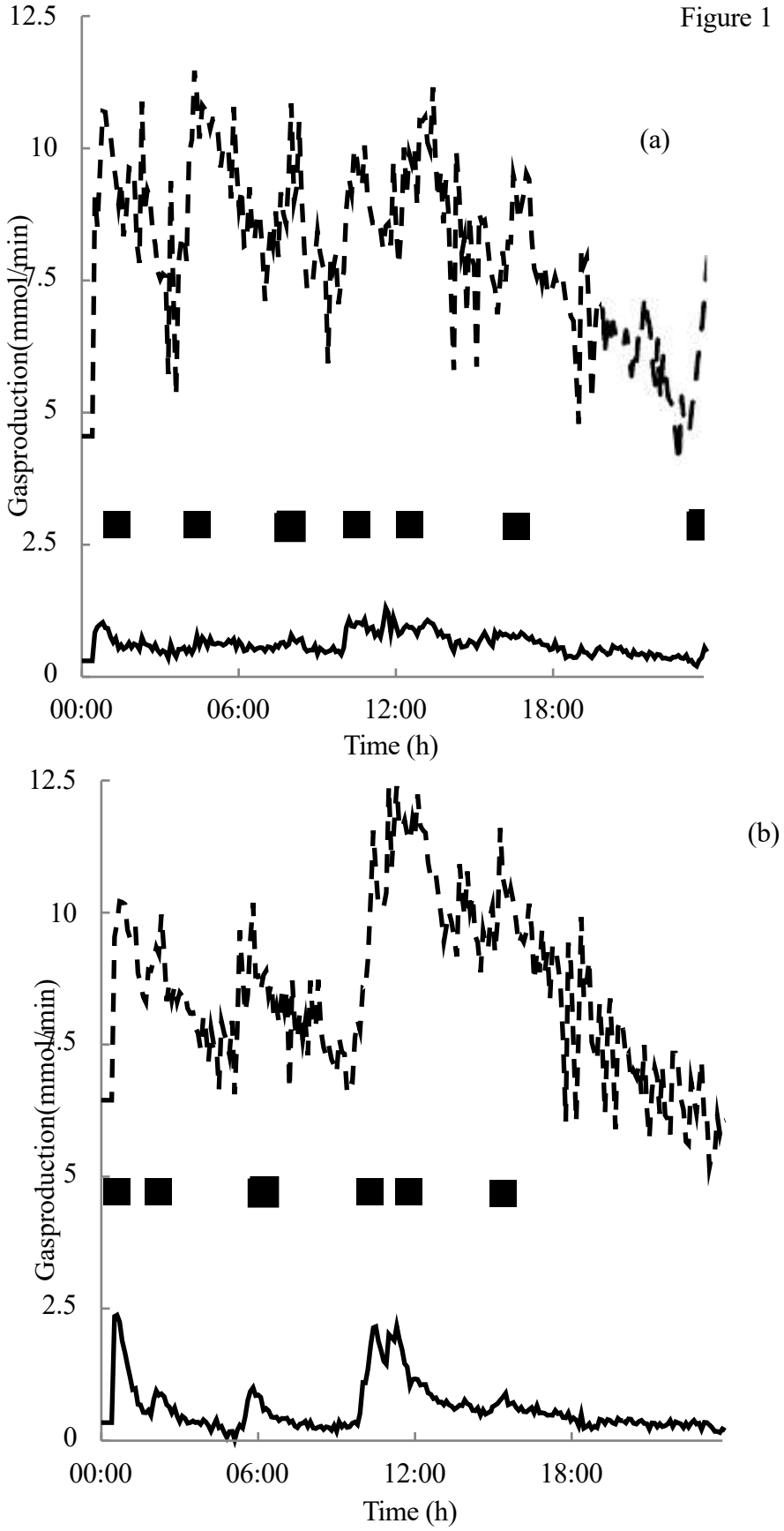
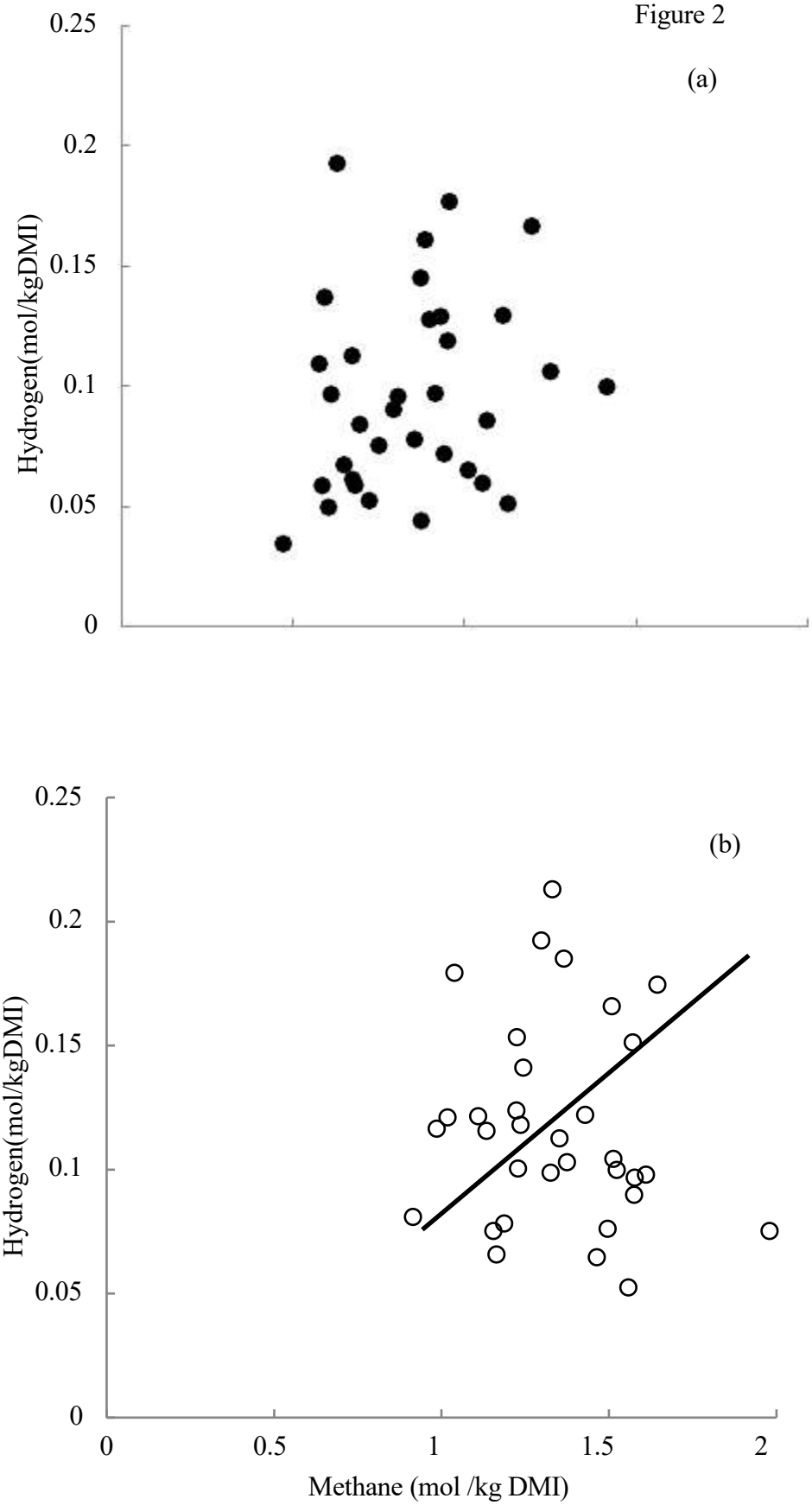

12.5
10
7.5
5
2.5
0
Gasproduction(mmol/min)
(a)
00:00
06:00
12:00
18:00
Time (h)
12.5
10
7.5
5
2.5
0
Gasproduction(mmol/min)
(b)
00:00
06:00
12:00
18:00
Time (h)

Figure 2


(a)
0.25
0.2
0.15
0.1
0.05
0
Hydrogen(mol/kgDMI)
(b)
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
Hydrogen(mol/kgDMI)
Methane (mol /kg DMI)